Issue of new matter

In regards to the issue of new matter, the applicant has adopted the claim format as suggested by the examiner. This overcomes the rejection of new matter.

Amendment of claim

- Canceling claim 1
- Amending claim and adopting new claims 4 and 5 as follow:

Claim 4. A quantitative method for molecular diagnosis of spinal muscular atrophy (SMA) comprising:

- growing a cell culture
- obtaining human samples containing the mRNA of survival motor neuron (SMN-mRNA) and the mRNA of human elongation factor 1-alpha (HUMEF1AB-mRNA) and control (normal subjects)
- reverse transcribing mRNA using primers consisting of SEQ ID NO:4 for the synthesis of cDNA from SMN-mRNA giving SMN-cDNA and SEQ ID NO:5 for the synthesis of cDNA from HUMEF1AB-mRNA giving HUMEF1AB-cDNA;

  measuring SMN-mRNA by means of BioImager device using the radioactive ^{32}p-dCTP labeled nucleotide probes SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3, wherein the probes are generated by PCR amplification of SEQ ID NO:10 and 11 for exon 7; SEQ ID NO:12 and SEQ ID NO:7 for exon 8 and, SEQ ID NO:8 and SEQ ID NO:9 for HUMEF1AB, comprising:

  - amplifying the SMN-cDNA by PCR using primers consisting of SEQ ID NO:6 and SEQ ID NO:7 and the HUMEF1AB-cDNA by PCR using primers consisting of SEQ ID NO:8 and SEQ ID NO:9
  - immobilizing of the obtained PCR products on the nylon membrane
  - hybridizing the immobilized PCR products with the radioactive

Contribution over the art

The state of the art is the qualitative diagnostic method of SMA at the DNA level. The applicant's contribution over the art is the quantitative diagnostic method at the mRNA level that permits the precise counting of the number of exons 7 and 8. The innovation is the use of specific probes to directly identify the presence or absence of exons 7 and 8: No one before has developed the method of synthesizing the probes specifically for SMA diagnostic purpose.

The point is not just to develop a quantitative molecular diagnostic method of SMA. The contribution over the art is (as described in the specification) the selection of the appropriate approach and combination of appropriate techniques that results in the development of this quantitative molecular diagnostic method of SMA which is safe, easy to handle and automated, easy to interpret, not costly and can be used widely in clinical laboratories. In addition, the contribution over the art is that the application offers two ways of measuring – by means of BioImager device using the radioactive labeled probes and by means of a microplate reader device in ELISA procedure using the biotin labeled probes; selecting which way depends on the type of equipment available at the laboratory.

Amendment of claim

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- reverse transcribing mRNA using primers consisting of SEQ ID NO:4 for the synthesis of cDNA from SMN-mRNA giving SMN-cDNA and SEQ ID NO:5 for the synthesis of cDNA from HUMEF1AB-mRNA giving HUMEF1AB-cDNA;

  measuring SMN-mRNA by means of BioImager device using the radioactive ^{32}p-dCTP labeled nucleotide probes SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3, wherein the probes are generated by PCR amplification of SEQ ID NO:10 and 11 for exon 7; SEQ ID NO:12 and SEQ ID NO:7 for exon 8 and, SEQ ID NO:8 and SEQ ID NO:9 for HUMEF1AB, comprising:

  - amplifying the SMN-cDNA by PCR using primers consisting of SEQ ID NO:6 and SEQ ID NO:7 and the HUMEF1AB-cDNA by PCR using primers consisting of SEQ ID NO:8 and SEQ ID NO:9
  - immobilizing of the obtained PCR products on the nylon membrane
  - hybridizing the immobilized PCR products with the radioactive

173, 147-153). Jong's work is an analysis in basic research using a semi-quantitative research method; it is not for diagnostic purpose.

The above-mentioned example shows that it is not obvious to identify an appropriate approach to develop a quantitative diagnostic method for SMA disease.

The applicant adopted a totally different approach:

Total RNA

↓
RT process using **SPECIFIC** primer (SEQ ID NO: 4) which recognizes the **SPECIFIC** SMN-mRNA; this results in **SMN-cDNA**

---------- SMN-cDNA
↓
PCR process using the **SPECIFIC** primers (SEQ ID NO: 6) and (SEQ ID NO: 7) to amplify the sequence fragments between exons 5 to 8

Exon 5 ---------- Exon 8
↓

Construction of the probes:
From the obtained fragments 5 to 8 by PCR:
- using the primers (SEQ ID NO:10) and (SEQ ID NO:11) to amplify a portion of exon 7
- using the primers (SEQ ID NO:12) and (SEQ ID NO:7) to amplify a portion of exon 8

The portion of exon 7 is labeled in two ways with radioactive label and with biotin label (non radioactive) to get probe 1 (SEQ ID NO:1)

The portion of exon 8 is labeled in two ways with radioactive label and with biotin label (non radioactive) to get probe 2 (SEQ ID NO:2)

The probes 1 and 2 are used to identify the presence or absence of exons 7 and 8 in normal subjects and SMA patients.

This applicant's quantitative diagnostic method is based on the use of SPECIFIC probes to DIRECTLY identify the presence or absence of exons 7 and 8 in different types of subjects. As of the date of the application, the applicant's quantitative SMA diagnostic method at the

mRNA level is the **only** diagnostic method that permits such precise counting of the number of exons 7 and 8 by measuring the **quantity** of radioactive rays emitted from the radioactive labeled probes, and by measuring the **intensity** of the coloration of the solution by using the biotin labeled probes in ELISA method. No one before has developed the method of synthesizing the probes specifically for SMA diagnostic purpose – probes directed to the deletion regions for the detection of the presence or absence of the nucleic acids (examiner acknowledges that Jong et al. do not teach this, p.11 Office Action).

How to quantify: Two ways of measuring

1/ Use of radioactive labeled probes (^{32}p dCTP labeled nucleotide probes) (pp. 4, 9, 10 Specification)

After immobilizing the PCR products on the nylon membrane, we perform the hybridization of the immobilized PCR products with the radioactive labeled probe 1 (SEQ ID NO:1) and probe 2 (SEQ ID NO:2). During this step, if the immobilized PCR products contain exon 7 and/or exon 8, the radioactive labeled probes 1 and/or 2 will bind to the immobilized PCR products at the position of exon 7 and/or exon 8. We thus detect the presence of the hybridized probes, which means the binding of the probes to the immobilized PCR products by autoradiography; we then quantify the amount of hybridized probes 1 and 2 by means of BioImager device by measuring the quantity of radioactivity rays emitted from the radioactive labeled probes 1 and 2. The quantity of radioactive rays emitted is proportional to the amount of radioactive labeled probes 1 and 2, and consequently proportional to the number of exon 7 and/or exon 8 present in the mRNA; this way allows us to count the number of exon 7 and/or 8 present in the mRNA.

2/ Use of biotin labeled probes in ELISA procedure for measurement of mRNA (pp. 4, 10, 11 Specification)

During the hybridization of the PCR products (containing the digoxigenin-dUTP) with the biotin labeled nucleotide probe 1 (SEQ ID NO:1) and probe 2 (SEQ ID NO:2), if the PCR products contain exon 7 and exon 8, the biotin labeled probes 1 and 2 will bind to the PCR products at the position of exon 7 and/or exon 8. Following this step, we perform the immobilization of the hybridized PCR products on streptavidin coated microtitration plates. During this step, the streptavidin will bind to the biotin molecules of the biotin labeled probes 1 and 2. Then, we add the peroxidase-conjugated anti-digoxigenin antibodies. This compound will bind to the digoxigenin-dUTP present in the hybridized PCR products. Then we add the peroxidase substrates which are H_2O_2 and chromogene (tetramethyl benzidine). We then quantify the amount of biotin-labeled probes 1 and 2 by means of a microplate reader device by measuring the transmitted light emitted from the chromogene (tetramethyl benzidine) which is one of the substrates of peroxidase enzyme. The quantity of transmitted light emitted from the chromogene is proportional to the amount of biotin labeled probes 1 and 2, and consequently proportional to the number of exon 7 and/or exon 8 present in the mRNA; this way thus allows us to count the number of exon 7 and/or exon 8 present in the mRNA.

As shown in the above description, it is not obvious to identify appropriate steps applicable for the selected approach in order to develop a quantitative diagnostic method for SMA disease. Jong's work arrived at a semi-quantitative method, not quantitative; and the purpose is not for diagnostic purpose.

The point is to determine what steps to take, then determine which techniques are needed to perform such tasks.

Amendment of claim

- Canceling claim 1
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Claim 4. A quantitative method for molecular diagnosis of spinal muscular atrophy (SMA) comprising:

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- obtaining human samples containing the mRNA of survival motor neuron (SMN-mRNA) and the mRNA of human elongation factor 1-alpha (HUMEF1AB-mRNA) and control (normal subjects)
- reverse transcribing mRNA using primers consisting of SEQ ID NO:4 for the synthesis of cDNA from SMN-mRNA giving SMN-cDNA and SEQ ID NO:5 for the synthesis of cDNA from HUMEF1AB-mRNA giving HUMEF1AB-cDNA;

  measuring SMN-mRNA by means of BioImager device using the radioactive ^{32}p-dCTP labeled nucleotide probes SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3, wherein the probes are generated by PCR amplification of SEQ ID NO:10 and 11 for exon 7; SEQ ID NO:12 and SEQ ID NO:7 for exon 8 and, SEQ ID NO:8 and SEQ ID NO:9 for HUMEF1AB, comprising:

  - amplifying the SMN-cDNA by PCR using primers consisting of SEQ ID NO:6 and SEQ ID NO:7 and the HUMEF1AB-cDNA by PCR using primers consisting of SEQ ID NO:8 and SEQ ID NO:9
  - immobilizing of the obtained PCR products on the nylon membrane
  - hybridizing the immobilized PCR products with the radioactive

^{32}p-dCTP labeled nucleotide probes SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3

- detecting the hybridized probes by autoradiography and quantifying the amount of SMN-mRNA by means of BioImager device.

Claim 5. A quantitative method for molecular diagnosis of spinal muscular atrophy (SMA) comprising:

- growing a cell culture
- obtaining human samples containing the mRNA of survival motor neuron (SMN-mRNA) and the mRNA of human elongation factor 1-alpha (HUMEF1AB-mRNA) and control (normal subjects)
- reverse transcribing mRNA using primers consisting of SEQ ID NO:4 for the synthesis of cDNA from SMN-mRNA giving SMN-cDNA and SEQ ID NO:5 for the synthesis of cDNA from HUMEF1AB-mRNA giving HUMEF1AB-cDNA;

measuring SMN-mRNA by means of microplate reader in ELISA procedure using the biotin-11-dCTP labeled nucleotide probes SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3, wherein the probes are generated by PCR amplification of SEQ ID NO:10 and 11 for exon 7; SEQ ID NO:12 and SEQ ID NO:7 for exon 8 and, SEQ ID NO:8 and SEQ ID NO:9 for HUMEF1AB, comprising:

- immobilizing of the streptavidin on the polystyrene microtitration plates
- amplifying the SMN-cDNA by PCR using primers consisting of SEQ ID NO:6 and SEQ ID NO:7 and the HUMEF1AB-cDNA by PCR using primers consisting of SEQ ID NO:8 and SEQ ID NO:9 in the presence of digoxigenin-11-dUTP

- hybridizing the obtained PCR products with the biotin-11-dCTP labeled nucleotide probes SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3
- immobilizing the hybridized products on the streptavidin coated microtitration plates
- adding the peroxidase-conjugated anti-digoxigenin antibodies
- adding the peroxidase substrates consisting of H_2O_2 and chromogene (tetramethyl benzidine)
- adding H_2SO_4 to stop the reaction
- reading the optical density (OD) for quantifying the amount of SMN-mRNA by means of microplate reader.